

CARDIOREGULATION IN LIMULUS. III. INHIBITION BY 5-HYDROXYTRYPTAMINE AND ANTAGONISM BY BROMLYSERGIC ACID DIETHYLAMIDE AND PICROTOXIN

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Cardioinhibition in *Limulus* appears to be chemically mediated. The decrease in heart rate resulting from stimulation of the inhibitor nerves is not tightly coupled to the stimulation, a time lag in the response occurring both at the beginning and at the end of the stimulation periods (Carlson, 1905; Pax and Sanborn, 1964).

The nature of the chemical mediator of inhibition is not known. A variety of pharmacological agents have been tested since the neurogenic nature of the heart beat was first shown by Carlson (1904). Of these, only three have been reported to cause a decrease in heart rate: ergot (Carlson, 1906), 5-hydroxytryptamine (5-HT) and gamma-aminobutyric acid (GABA) (Burgen and Kuffler, 1957).

A study of GABA as the possible inhibitory neurotransmitter in the *Limulus* heart has previously been reported (Pax and Sanborn, 1967). Although this compound decreases rate and strength of beating when applied artificially, it does not decrease the number of units discharging or the total duration of each burst of electrical activity in the cardiac ganglion as does stimulation of the inhibitor nerves. Moreover, picrotoxin, though effective in blocking the function of the inhibitor nerves, is not an effective antagonist to GABA activity. It appears, therefore, that GABA is not involved as the inhibitory neurotransmitter in the *Limulus* heart.

5-HT, like GABA, is found in a wide variety of animals (Welsh and Moorhead, 1960). It has been shown to have physiological significance in such diverse animal groups as flatworms and vertebrates (Mansour *et al.*, 1960). In contrast to its reported inhibitory action on the *Limulus* heart (Burgen and Kuffler, 1957) it has an excitatory effect on the crustacean neurogenic heart (Florey and Florey, 1954; Maynard and Welsh, 1959; Kerkut and Price, 1964; Cooke, 1966). We report here results of experiments exploring more fully the possibility that 5-HT or a 5-HT-like compound is the cardioinhibitory transmitter in the neurogenic *Limulus* heart.

MATERIALS AND METHODS

Materials and methods are as previously described (Pax and Sanborn, 1967).

RESULTS

5-Hydroxytryptamine

Perfusion of 5-HT through the isolated heart results in a decrease in heart rate. A typical result of 5-HT perfusion is shown in Figure 1. In Figure 2 the

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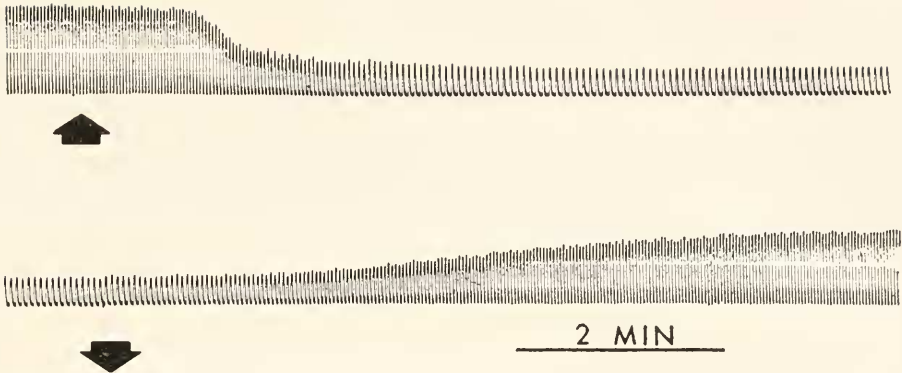


FIGURE 1. Response of the isolated heart to perfusion of 5-HT. During the time between the arrows 100 ml. of 5×10^{-6} M 5-HT were perfused through the heart.

relationship between concentration of 5-HT perfused and relative heart rate is plotted for 18 perfusions of 5-HT in 12 different hearts. The solid line on the graph is the regression line for these data as determined by the method of least squares. The standard error of this line is indicated by the dashed lines on either side of the regression line. The slope of this regression line is -0.34 ; the standard error 0.17. The threshold for rate changes, as determined by solving the equation

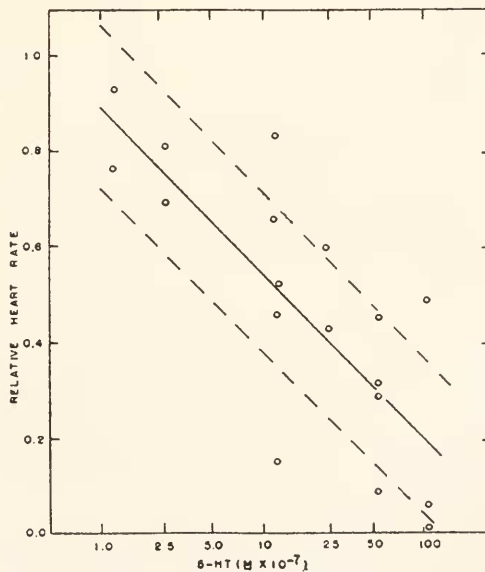


FIGURE 2. Relation of relative heart rate to concentration of 5-HT perfused through the isolated heart. Each point represents a single perfusion. The solid line is the regression line determined by the method of least squares and the dashed line is the standard error of the regression line.

for the regression line, is $4.9 \times 10^{-8} M$ while at $4.1 \times 10^{-5} M$ 5-HT a relative heart rate of zero would be expected.

The strength of heart beat also decreases when 5-HT is perfused through the isolated heart (Fig. 1). In Figure 3 the relationship between 5-HT concentration and relative contraction strength is plotted for 14 perfusions of 5-HT in nine different hearts. The calculated threshold concentration for strength changes is $5.6 \times 10^{-8} M$, about the same as that calculated for rate changes, but the calculated regression lines for rate and strength changes are not parallel (slope = -0.34 for rate, -0.31 for strength).

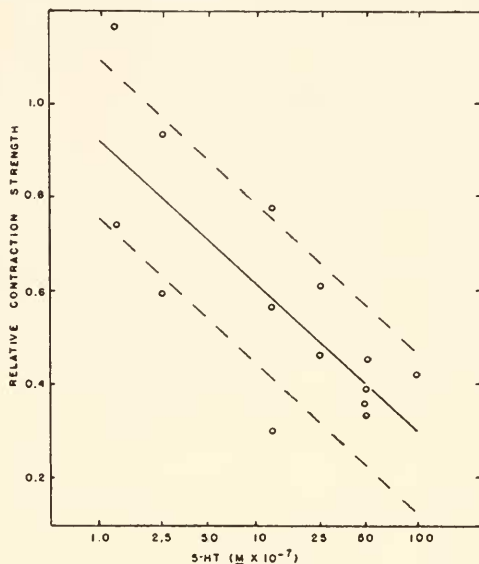


FIGURE 3. Relation of relative contraction strength to concentration of 5-HT perfused through the isolated heart. Each point represents a single perfusion. The solid line is the regression line determined by the method of least squares and the dashed lines are the standard error of the regression line.

Both of the above effects of 5-HT are readily reversible. Perfusion with drug-free saline for five minutes following drug treatment is usually sufficient to bring the rate and strength of beating within 10% of their pre-treatment levels.

Neither 5-hydroxytryptophan—the precursor of 5-HT—nor 5-hydroxyindoleacetic acid—its major metabolite—at $10^{-4} M$ had any detectable effects on rate or strength of beating of the isolated heart.

Electrical activity of the isolated cardiac ganglion is also affected by 5-HT treatment. The rate of rhythmic discharges decreases. The number of units discharging in each burst is reduced and the total duration of each burst is lessened. The pattern of a typical burst of electrical activity recorded from the fourth segment of the isolated cardiac ganglion before treatment and after treatment with $1 \times 10^{-6} M$ 5-HT is shown in Figure 4. In this experiment the relative heart rate during

perfusion of 5-HT was 0.51. The changes in the pattern of electrical activity in the ganglion during a particular burst are readily apparent.

After treatment of isolated ganglia with 5-HT, one minute of bathing in drug-free saline returns the rate to the pre-treatment level.

Bromlysergic acid diethylamide

Bromlysergic acid diethylamide (BOL) is a potent and specific antagonist of 5-HT in other animals (Gyermek, 1961). Since 5-HT appears to mimic the action of the inhibitor nerves of the *Limulus* heart we have studied the interaction between BOL and the inhibitor nerves. The function of the inhibitor nerves was tested in four animals before, during, and after perfusion with 1.6×10^{-5} M BOL. These experiments were performed in a manner parallel to that used in testing the interaction of the inhibitor nerves and picrotoxin (Pax and Sanborn, 1967). Nerves were stimulated for 20 seconds out of every five minutes. During the first five

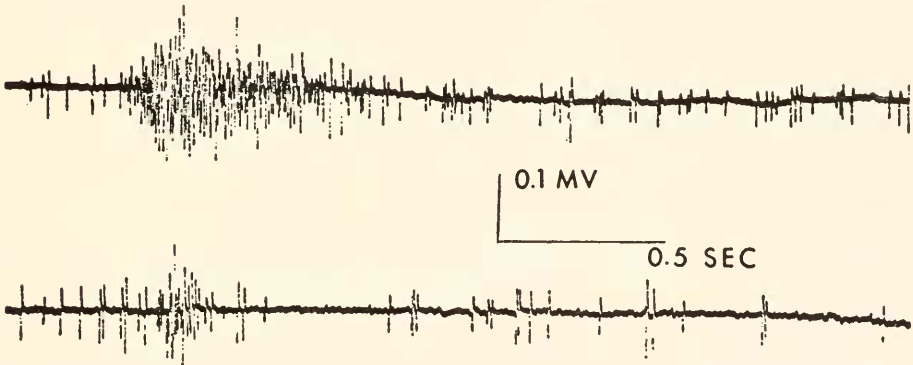


FIGURE 4. Changes in the pattern of electrical activity in the isolated ganglion resulting from 5-HT treatment. The upper trace is a representative burst before drug treatment, the lower trace a representative burst after bathing the ganglion for one minute in 1×10^{-6} M 5-HT.

five-minute stimulation intervals, drug-free saline was perfused. During the next two five-minute intervals 100 ml. of BOL were perfused and during the last six five-minute intervals drug-free saline was again perfused.

BOL (1.6×10^{-5} M) alone causes a slight increase in heart rate, the mean rate for 15 different hearts being 30.3 beats per minute before BOL treatment and 31.8 beats per minute after BOL treatment. The relative rate for each of the 13 stimulation periods was computed by taking the ratio of the rate during the stimulation period to the rate just previous to that same stimulation period.

BOL is an effective antagonist of inhibitor nerve action in the *Limulus* heart. The mean relative rate during stimulation of the inhibitor nerves for each of the 13 stimulation periods is shown in Figure 5. Stimulation of the inhibitor nerves before BOL treatment resulted in a mean decrease in rate of 19.8 beats per minute. The relative rate was 0.28 (SD = 0.11), *i.e.*, stimulation reduced the rate by 72%. After BOL treatment the decrease in rate was 12.0 beats per minute and the relative rate was 0.66 (SD = 0.12), *i.e.*, stimulation reduced the rate by only 34%.

A "t" test for the difference between the two relative rates showed the inhibitor nerves significantly less effective in decreasing heart rate after BOL treatment ($P > 0.95$). Function of the inhibitor nerves does not begin to return to the pre-BOL perfusion level even after perfusion with drug-free saline for as long as 30 minutes (Fig. 5).

Since BOL blocks the function of the cardioinhibitory nerves in *Limulus*, it should also antagonize the action of artificially applied 5-HT, if 5-HT is acting at a junction in the cardioinhibitory pathway. The ability of BOL to antagonize the action of 5-HT was tested on four isolated hearts.

These experiments were performed in a manner parallel to our experiments testing the interaction of GABA and picrotoxin. One hundred ml. of saline con-

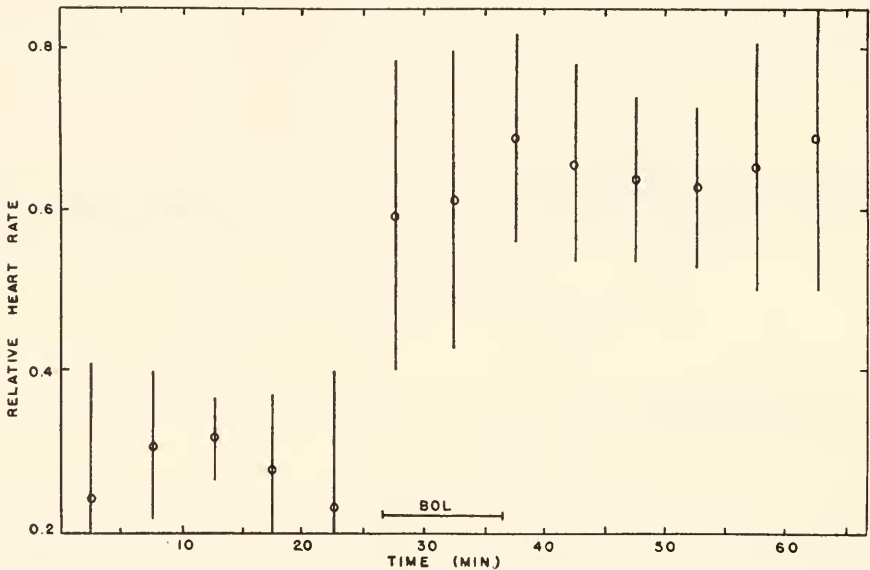


FIGURE 5. Effect of BOL on the function of the cardioinhibitory nerves. Each point represents the mean relative heart rate for four hearts during stimulation of the inhibitor nerves. Vertical lines extend one standard deviation on either side of the mean. During the time between the two arrows 100 ml. of $1.6 \times 10^{-5} M$ BOL were perfused.

taining 5-HT were initially perfused through each heart to calibrate its response. After one-half hour of perfusion with drug-free saline to eliminate the effects of the 5-HT, 100 ml. of $1.6 \times 10^{-5} M$ BOL were perfused. This perfusion was immediately followed by perfusion of 100 ml. of $1.6 \times 10^{-5} M$ BOL to which had been added the same concentration of 5-HT as that previously given during the calibration perfusion. 5-HT at concentrations of 1 and $5 \times 10^{-6} M$ was tested in this way against BOL at $1.6 \times 10^{-5} M$.

Our experiments show that this concentration of BOL is an effective antagonist of the rate-decreasing effects of 5-HT. The mean relative rate with 5-HT perfusion prior to BOL treatment was 0.57 (mean decrease in rate, 12.9 beats per minute) while after BOL treatment it was 0.93 (mean decrease in rate, 2.1 beats per

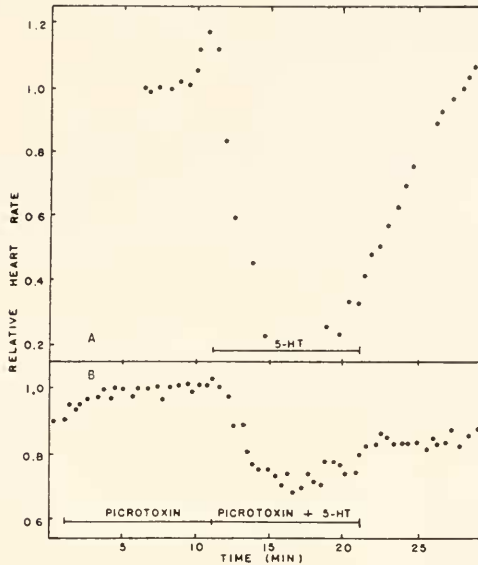


FIGURE 6. Rate changes in a heart perfused with 5×10^{-6} M 5-HT alone and with 5-HT plus 1.6×10^{-5} M BOL. See text for details.

minute). A “*t*” test for the difference between the two relative rates showed 5-HT significantly less effective in reducing heart rate after BOL treatment ($P > 0.99$). The results of a typical experiment are shown in Figure 6.

BOL (1.6×10^{-5} M) is also an effective antagonist of the strength-decreasing effects of artificially applied 5-HT. The mean relative strength with 5-HT perfusion prior to BOL treatment was 0.41 while after BOL treatment it was 1.04. A

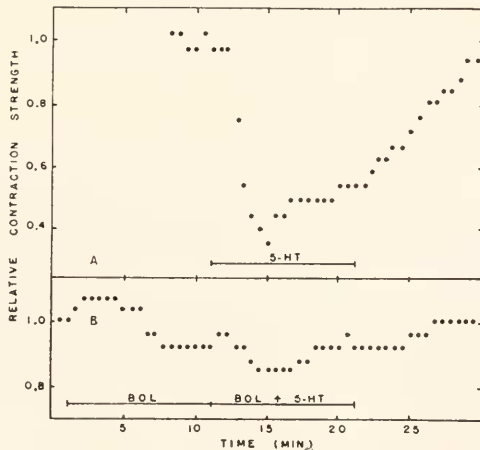


FIGURE 7. Changes in contraction strength with perfusion of 5×10^{-6} M 5-HT alone and with 5-HT plus 1.6×10^{-5} M BOL. See text for details.

"*t*" test for the difference between these means showed it significant ($P > 0.99$). Results of a typical experiment are shown in Figure 7.

Picrotoxin

Picrotoxin has previously been shown to be effective in blocking the action of the inhibitor nerves in *Limulus* (Pax and Sanborn, 1967). Since it blocks the inhibitor nerves it should also antagonize the action of applied 5-HT, if 5-HT is acting as a neurotransmitter in the cardioinhibitory pathway. We have therefore tested the ability of picrotoxin to block the action of applied 5-HT on four isolated hearts.

The experiments were performed in a manner parallel to that described for testing the interaction of 5-HT and BOL. One hundred ml. of saline containing 5-HT were initially perfused through each heart to determine a control response. After one-half hour of perfusion with drug-free saline to eliminate the effects of

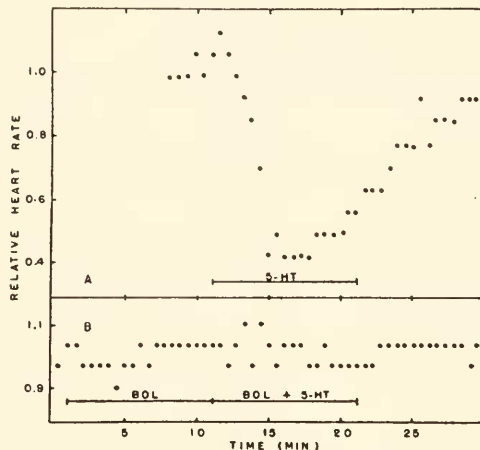


FIGURE 8. Rate changes in a heart perfused with $5 \times 10^{-6} M$ 5-HT alone and with 5-HT plus $1 \times 10^{-3} M$ picrotoxin. See text for details.

the 5-HT, 100 ml. of $10^{-3} M$ picrotoxin were perfused followed immediately by 100 ml. of $10^{-3} M$ picrotoxin to which had been added the same concentration of 5-HT as that given during the control perfusion. 5-HT at concentrations of 1, 5, and $10 \times 10^{-6} M$ was tested in this way against picrotoxin at $10^{-3} M$.

As with BOL, there is antagonism between 5-HT and picrotoxin. The mean relative rate with 5-HT perfusion prior to picrotoxin treatment was 0.34 (mean decrease in rate, 18.2 beats per minute) while after picrotoxin treatment it was 0.75 (mean decrease in rate, 11.5 beats per minute). A "*t*" test for the difference between the two relative rates showed the relative rate to be significantly higher after picrotoxin treatment ($P > 0.99$). The results of a typical experiment are presented in Figure 8.

By contrast, the effects upon relative contraction strength are quite different. In this variable, picrotoxin and 5-HT show synergism rather than antagonism.

Though no measurable change in contraction strength is brought about by perfusion of picrotoxin alone, when picrotoxin is perfused with 5-HT a greater decrease in contraction strength occurs than when 5-HT alone is perfused. The mean relative strength of four hearts with 5-HT perfusion prior to picrotoxin treatment was 0.51 while after picrotoxin treatment it was 0.26. A "t" test for the difference between these means showed the relative contraction strength significantly lower after picrotoxin treatment than before ($P > 0.95$). The results of a typical experiment are presented in Figure 9.

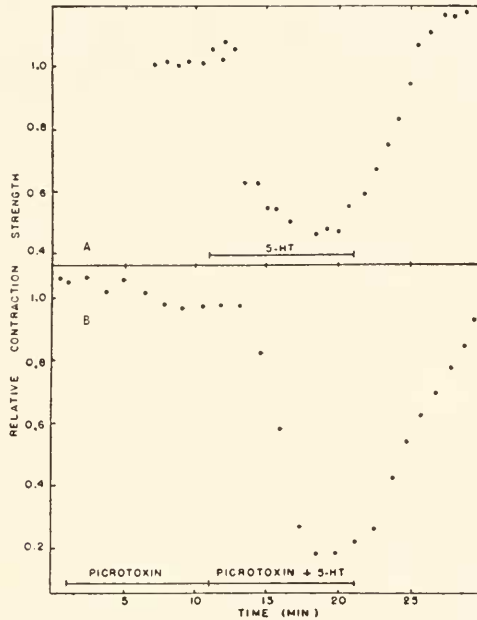


FIGURE 9. Changes in contraction strength with perfusion of 5×10^{-6} M 5-HT alone and with 5-HT plus 1×10^{-3} M picrotoxin. See text for details.

BOL and GABA

BOL blocks the function of the cardioinhibitory nerves. GABA, although it decreases the rate and strength of beating of the intact heart, does not alter the pattern of electrical activity in the cardiac ganglion. Thus, GABA does not mimic stimulation of the inhibitor nerves and appears to produce its effects at some site other than the cardioinhibitory pathway in the *Limulus* heart (Pax and Sanborn, 1967). If BOL is blocking the action of the inhibitor nerves by acting specifically at a junction in the cardioinhibitory pathway, and GABA is acting at some site other than this, then there should be no interaction between simultaneously applied BOL and GABA.

We have tested this in eight isolated hearts. One hundred ml. of saline containing GABA were initially perfused through each heart. After one-half hour of perfusion with drug-free saline, 100 ml. of 1.6×10^{-5} M BOL were perfused. This

was followed immediately by 100 ml. of 1.6×10^{-5} M BOL to which had been added the concentration of GABA previously given during the control perfusion. GABA at concentrations of 5, 10, and 50×10^{-6} M was tested in this way against BOL at 1.6×10^{-5} M.

There is no apparent interaction between BOL and GABA so far as rate is concerned. The mean relative rate resulting from GABA perfusion prior to BOL treatment was 0.34 (mean decrease in rate, 19.7 beats per minute) while after BOL treatment it was 0.44 (mean decrease in rate, 15.4 beats per minute). In four out of eight hearts tested in this manner the relative rate resulting from GABA perfusion was higher after BOL treatment than before. In the other four it was lower. The mean difference between the relative rate prior to, and following BOL treatment was 0.10 (SD = 0.18). A "t" test for the difference between the relative rate before BOL treatment and after BOL treatment showed it to be non-significant ($P < 0.90$).

TABLE I
Summary of the responses of the *Limulus* heart to treatment
with various inhibitors and antagonists

Inhibitor	5-HT	5-HT	GABA
Antagonist	BOL	Picrotoxin	BOL
Mean relative rate			
No. animals tested	4	4	8
Inhibitor alone	0.57	0.34	0.34
Inhibitor with antagonist	0.93	0.75	0.44
Difference \pm 1 SD	0.36 ± 0.09	0.41 ± 0.18	0.10 ± 0.19
	$P < 0.01$	$P < 0.01$	$0.10 < P < 0.20$
Mean relative contraction strength			
No. animals tested	4	4	5
Inhibitor alone	0.41	0.51	0.67
Inhibitor with antagonist	1.04	0.26	0.65
Difference \pm 1 SD	0.63 ± 0.21	-0.25 ± 0.11	-0.02 ± 0.07
	$P < 0.01$	$0.01 < P < 0.05$	$0.60 < P < 0.70$

Similarly, there is no apparent interaction between BOL and GABA with respect to strength of contraction. For five hearts the relative contraction strength resulting from GABA perfusion prior to BOL treatment was 0.67 while after BOL treatment it was 0.65. A "t" test for the difference between these means showed it not significant ($P < 0.90$).

The results of the various treatments are summarized in Table I.

DISCUSSION

In the first paper of this series (Pax and Sanborn, 1964), we presented our reasons for believing that a chemical transmitter was involved in the inhibition of the neurogenic *Limulus* heart. At this point it is appropriate to examine the known and possible components of the *Limulus* cardioinhibitory system in order to visualize the sites at which chemical transmitters might operate.

Both the decapod crustacean cardiac ganglion and the *Limulus* cardiac ganglion possess two cell types (Heinbecker, 1936). The primary difference between the *Limulus* heart and the crustacean heart appears to be in the number of cells involved and it would seem reasonable to assume that the mechanism by which the rhythmic discharge is originated is common to both hearts (Maynard, 1955).

In the crustacean cardiac ganglion the burst is usually initiated by the smaller cells, the pacemakers. The larger cells are the major motor neurons (followers) and appear to be only relays which increase the number of impulses. Feedback, if any, from the followers to the pacemakers is small since only long subthreshold current pulses to the followers or a long series of follower cell impulses are necessary for modification of the rhythm of the pacemaker cells (Otani and Bullock, 1959). The inhibitor fibers make connections with both the pacemaker and the follower cells (Terzuolo and Bullock, 1958).

If the *Limulus* cardiac ganglion has an arrangement of functional units similar to the decapod heart, then we may diagram the inhibitory pathway as in Figure 10. Spontaneous rhythmic activity in the pacemaker cell (P) produces postsynaptic

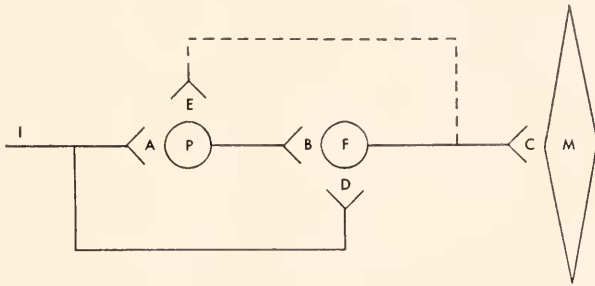


FIGURE 10. Diagram showing possible organization of the nerve cells and the cardioinhibitory connections in the cardiac ganglion of *Limulus*. Symbols explained in the text.

potentials in the follower cell (F). These postsynaptic potentials result in propagated action potentials which produce contraction of the myocardium (M). A block in transmission across the neuromuscular junction (C) would result in only a decreased strength of contraction. A block in transmission at junction "B" would give the same results.

Activity in the inhibitor nerve through its action at junction "A" would produce a decrease in the rate of spontaneous bursting in cell "P" and thus cause a decrease in heart beat rate. Some lesser effect on contraction strength might occur if activity in the inhibitor also lessens the number of discharges in the pacemaker during any particular burst of activity. At junction "D," activity in the inhibitor nerve would produce a decrease in contraction strength by reducing the number of action potentials in the follower cell.

Turning now to the results of the experiments described here we find that 5-HT decreases both rate and strength of beating. This could be due to activation of junction "A" alone or of both junction "A" and "D." BOL blocks the action of the inhibitor nerves but does not otherwise disrupt heart function and thus probably acts at junction "A" alone or at both "A" and "D." Since this compound also

blocks the action of applied 5-HT it appears probable that 5-HT acts at these same junctions.

Picrotoxin also blocks the action of the inhibitor nerves without otherwise markedly disrupting heart function. Thus it probably also acts at junction "A" or both "A" and "D." The rate-decreasing action of 5-HT is blocked by picrotoxin so again it would appear that 5-HT is acting at these same junctions. In contrast to the antagonism shown between BOL and 5-HT as far as strength-changing abilities are concerned, picrotoxin enhances the strength-decreasing ability of 5-HT. Such a pattern of responses could occur if junction "D" possesses pharmacological properties which are slightly different from those of junction "A."

GABA interacts with neither picrotoxin nor BOL. Thus it appears to act at neither junction "A" nor "D." It probably does not act at junction "B" or "C" since the rate-reducing effects of GABA are produced in the isolated ganglion and no change in burst parameters is noted with application of GABA. At this time we have no way of assessing the significance of feedback from the followers to the pacemakers (dashed line and junction "E" in Figure 10). Perhaps the major site of action of GABA is in this pathway.

Whether the endogenous inhibitory transmitter of the heart of *Limulus* is 5-HT or some related compound is open to question. Cogeners of 5-HT such as 5,6-dihydroxytryptamine, 6-hydroxytryptamine, or other substituted hydroxytryptamines have not been tested and may be as potent as 5-HT. In the crustacean heart 5-HT, 5,6-dihydroxytryptamine and 6-hydroxytryptamine are all potent cardiotropic agents and all have been detected in tissue extracts (Carlisle, 1956; Maynard and Welsh, 1959; Kerkut and Price, 1964; Belamarich and Terwilliger, 1966).

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SUMMARY

1. Heart rate in *Limulus* is slowed by 5-hydroxytryptamine (5-HT). The threshold for this inhibition is 4.9×10^{-8} M.
2. The strength of beat is also reduced in 5-HT solutions. The calculated threshold for this effect is 5.6×10^{-8} M.
3. Both of these effects are readily reversible.
4. Neither 5-hydroxytryptophan (10^{-4} M) or 5-hydroxyindole acetic acid (10^{-4} M) have any detectable effects on rate or strength of beating.
5. Applied to the isolated cardiac ganglion, 5-hydroxytryptamine (10^{-6} M) decreases the rate of rhythmic discharge, reduces the number of neurons discharging in each burst, and lessens the duration of each burst. All of these effects are also reversible.
6. Bromlysergic acid diethylamide (BOL), 1.6×10^{-5} M, decreases the ability of the cardiainhibitory nerves to influence heart rate.
7. BOL prevents the rate and strength changes engendered by exogenous 5-HT applied to the isolated heart.

8. Picrotoxin antagonizes the decrease in heart rate produced by application of 5-HT, but synergizes with 5-HT with respect to its strength-decreasing ability.

9. No interaction between BOL and γ -aminobutyric acid (GABA) could be demonstrated.

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